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REVIEW

Enhancing the utility of existing antibiotics by targeting bacterial behaviour?

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The discovery of novel classes of antibiotics has slowed dramatically. This has occurred during a time when the appearance of resistant strains of bacteria has shown a substantial increase. Concern is therefore mounting over our ability to continue to treat infections in an effective manner using the antibiotics that are currently available. While ongoing efforts to discover new antibiotics are important, these must be coupled with strategies that aim to maintain as far as possible the spectrum of activity of existing antibiotics. In many instances, the resistance to antibiotics exhibited by bacteria in chronic infections is mediated not by direct resistance mechanisms, but by the adoption of modes of growth that confer reduced susceptibility. These include the formation of biofilms and the occurrence of subpopulations of 'persister' cells. As our understanding of these processes has increased, a number of new potential drug targets have been revealed. Here, advances in our ability to disrupt these systems that confer reduced susceptibility, and in turn increase the efficacy of antibiotic therapy, are discussed.

Abbreviations

AHL, *N*-acyl-homoserine lactone; AIP, autoinducing peptide; AIs, autoinducers; c-di-GMP, cyclic di-GMP; KPC, *Klebsiella pneumoniae* carbapenemases; MIC, minimum inhibitory concentration; NDM-1, New Delhi metallo-β-lactamase-1; PNAG, poly-β-1, 6-N-acetylglucosamine; QQ, quorum quenching; QS, quorum sensing; QSI, quorum-sensing inhibitor; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; SAM, S-adenosyl methionine; TA, toxin–antitoxin; VIM, Verona integron-encoded metallo-β-lactamase

A world without antibiotics?

The discovery and development of antibiotics undoubtedly ranks among the greatest achievements of medicine. The routine use of antibiotics to treat bacterial infections for more than 65 years makes life without them difficult to imagine, and it can be easily forgotten that before the introduction of antibiotics, deaths resulting from minor wound infections by pathogens such as *Staphylococcus aureus* were commonplace.

These years of antibiotic use, some of which has been indiscriminate and unnecessary (Cusini *et al.*, 2010), has led to the emergence of bacteria able to resist many commonly used antibiotics (Fischbach and Walsh, 2009; IDSA, 2004; CDC, 2006). In addition, many different forms of antibiotic resistance mechanisms can move by horizontal gene transfer to previously susceptible cells of the same, or worse, different species. The data on the human impact of antibiotic resistance are unsettling. Research from the Centers for Disease Control and Prevention (CDC) has shown that antibiotic use, and in turn the occurrence of resistant strains of bacteria, have increased dramatically over the past two decades (Bartlett, 1999). According to the Infectious Diseases

Society of America (CDC, 2006), two million people suffer bacterial infections each year, with 90 000 resultant fatalities (CDC, 2006). Of these, approximately 70% are due to organisms that are resistant to at least one antibiotic (CDC, 2006). The potential impact of growing levels of resistance was reflected in the World Health Organization devoting the World Health Day on 7 April 2011 to raising awareness of this issue.

In many countries, the UK included (Dryden *et al.*, 2009), access to antibiotics is controlled. The spread of antibiotic resistant strains globally undermines these efforts. Here, the fact that many of the drug-resistant Gram-positive strains encountered in hospitals worldwide are resistant to even supposedly last-line agents, such as vancomycin (CDC, 2006; Talbot *et al.*, 2006), is a serious cause for concern. For example, the UK sees the repeated import of strains of Enterobacteriaceae, a family of bacteria that includes many species that are part of the normal gut microbiota, with Verona integron encoded (VIM) metallo-carbapenemases and *Klebsiella pneumoniae* carbapenemases (KPC; class A) via patients previously hospitalized in Greece, Cyprus and Israel (Vatopoulos, 2008; Kitchel *et al.*, 2009; Nordmann *et al.*, 2009; Livermore *et al.*, 2011). Recently, the emergence of bacterial species carrying a gene for a novel enzyme, New Delhi metallo β-lactamase (NDM-1) has been causing increasing concern (Kumarasamy *et al.*, 2010; Livermore *et al.*, 2011). A patient, repatriated to Sweden after admission to hospital in New Delhi, India, was colonized by *K. pneumoniae* and *Escherichia coli* with plasmid-borne NDM-1 genes (Yong *et al.*, 2009). The distribution of these genes has since been shown to be widespread in India and Pakistan and represent a significant threat through importation into the UK by previously hospitalized patients returning from the Indian subcontinent (Kumarasamy *et al.*, 2010; Livermore *et al.*, 2011).

Drivers of resistance

The evolution of antibiotic resistance can be seen as a relatively simple case of adaptation by natural selection. Exposure of bacteria to antibiotics drives resistance by selecting for those cells that are able to tolerate them. As such, there are strongly positive correlations between the level of antibiotic use and the prevalence of antibiotic-resistant bacteria in the same human population, both at national and regional levels (Hogberg *et al.*, 2010, and references therein). The rate of emergence of resistance is related to total antibiotic use, including, for example, the widespread prescription of antibiotics in response to respiratory viral infection (Ben-David and Rubinstein, 2002). Further, in some regions, particularly in the developing world, access to antibiotics can be almost entirely unregulated (Hart and Kariuki, 1998; Zhan *et al.*, 1998). Exposure of bacteria to antibiotics comes in other ways. Low-dose antibiotics, as a total representing 40 and 80% of all antibiotics used in America are given to promote growth in livestock (Shea *et al.*, 2004; Food and Drug Administration, Department of Health and Human Services, 2009). While this practice is outlawed within the European Union, the capacity for resistance spread makes this a global issue. Of particular importance here is the cross-resistance that can develop to antibiotics used widely in livestock and those that are critically important in human medicine, for example, between apramycin and gentamicin (Chaslus-Dancla *et al.*, 1991). Even where bacterial prescription is appropriate, a failure to achieve sufficient levels at the site of infection, or for a sufficient duration, can also accelerate the emergence of resistance.

Concerted efforts are being made to reduce the rate at which resistant bacterial strains are appearing, for example, by employing better antimicrobial stewardship (MacDougall and Polk, 2005; McGregor *et al.*, 2006; Dellit *et al.*, 2007; Patrick and Hutchinson, 2009; Sabuncu *et al.*, 2009; Cooke *et al.*, 2010; Grigoryan *et al.*, 2010). However, such measures can only be partially effective, and are particularly difficult to implement in the developing world. Regardless, resistance is a natural and unavoidable consequence of antibiotic use. In response to growing concern about our decreasing ability to treat effectively bacterial infections with the antibiotics available to us, a number of review articles have argued that efforts to develop novel antibiotics need to be redoubled. Indeed, Coates *et al.* argued strongly in the *British Journal of Pharmacology* recently that we need to develop not just novel antibiotics but novel classes of antibiotics (Coates *et al.*, 2011).

The search for new antibiotics

Known classes of antibiotics represent a relatively small set of chemical scaffolds that have been modified over the past few decades by synthetic tailoring into new-generation agents (Fernandes, 2006; Fischbach and Walsh, 2009) that generally offer incremental improvements over existing therapies and typically expand the spectrum of use. The scaffolds on which these changes are made can be either natural compounds, largely discovered by empirical screening, such as 6-aminopenicillanic acid, from which analogues such as amoxicillin have been developed (Rolinson and Geddes, 2007), or synthetic compounds, for instance, protonsil, which was the precursor of sulpha drugs (metronidazole, isoniazid and oxazolidinones). However, the number of analogues that can be developed from a given molecule is limited.

The calls for greater efforts to identify new classes of antibiotics, as set out by Coates *et al.* (2011) among others, are fully justified, and the various strategies that are being employed in the search for novel antibiotics has been the subject of a number of comprehensive reviews (Coates and Hu, 2007; Davies, 2011). However, there has been a decline in new antibiotic approvals over the past 20 years (Spellberg *et al.*, 2004), with their appearance currently being outstripped by the rate at which resistant strains are emerging (CDC, 2006; Spellberg *et al.*, 2004; Talbot *et al.*, 2006). Certainly too, a major breakthrough in the availability of novel antibiotic classes cannot be expected in the near future because very few antibacterial agents have entered clinical development in recent years (Butler and Cooper, 2011; Rogers *et al.*, 2011).

The difficulty of identifying new antibiotics is compounded by the increasing reluctance of pharmaceutical companies to invest the significant sums necessary to bring

drugs to market. The pharmaceutical research and development costs, estimated to be £240–480 million per approved agent (DiMasi *et al.*, 2003), pose a considerable barrier to new drug development in general, with pharmaceutical companies perceiving a better return in focusing on the development of drugs, for example, for chronic medical conditions that are more prevalent among the increasing elderly population in western societies, such as hypercholesterolaemia, hypertension, mood disorders, dementia and arthritis (Spellberg *et al.*, 2004). Further, once they reach market, novel antibiotics face stiff competition from already approved antimicrobials, as well as a reluctance by practitioners to deploy widely new antibiotics to which resistance is yet to develop. These factors have led to the pharmaceutical industry greatly reducing their involvement in anti-infectives research (Gilbert and Edwards, 2002; Shlaes and Moellering, 2002; Projan, 2003; Wenzel, 2004; Davies, 2006). Clearly, therefore, in order to treat infections effectively, new ways to use existing antibiotics must be developed.

Non-antibiotic pharmaceuticals that enhance the effectiveness of existing antibiotics

One way in which the useful life of existing antibiotics can be increased might be to use them in combination with non-antibiotic drugs that, for example, limit the ability of otherwise resistant bacteria to degrade or inactivate them. Since the 1950s, it has been recognized that it is possible to increase antibiotic impact by co-administration with certain non-antibiotic pharmaceuticals; for example, potassium clavulanate in the case in co-amoxiclav. Co-amoxiclav contains amoxicillin trihydrate, a β-lactam antibiotic, with potassium clavulanate, a β-lactamase inhibitor. This combination results in an increased spectrum of action and restored efficacy against β-lactamase-producing amoxicillin-resistant cells. More recently, NXL104, a novel β-lactamase inhibitor, has been shown to increase the efficacy of ceftazidime in the treatment of infection by resistant organisms (Endimiani *et al.*, 2011; Livermore *et al.*, 2011). This search continues. In many instances, multidrug resistant phenotypes are the result of overexpressed efflux pumps. Here, compounds that are efflux pump inhibitors such as chlorpromazine, amitryptiline and *trans*-chlorprothixene, have been shown to reduce or reverse resistance to antibiotics (Kristiansen *et al.*, 2010).

While it is clearly of great benefit that antibiotics can be 'protected' in this way, this is not always possible. Further, while such combination treatments may serve to mitigate against the spread of antibiotic resistance, we propose that there are translational benefits to be gained too from obtaining insights into how pathogens behave at the site of infection.

New opportunities?

An effective way to limit the emergence of resistance and preserve the usefulness of currently available antibiotic drugs would be to limit exposure of microbes to them. Were it possible to deliver lower doses of antibiotics, for shorter periods, while maintaining the same antimicrobial impact, the rate at which resistance would emerge and spread might be reduced. This would provide some respite in the inevitable emergence of resistance and allow time for new antibiotics to be developed. Fortunately, the recent rapid expansion in our understanding of how bacteria behave in infection, and their responses to both host immune systems and antimicrobial therapies, means that we are now in a position to re-evaluate antibacterial therapy and develop more effective approaches to treatment. This progress has been due in a large part to two factors: the emergence of molecular genetic tools that provide the ability to determine directly the manner in which bacteria behave at the site of infections and the development of more sophisticated *in vivo* and *in vitro* model systems. Application of these tools has allowed us for the first time to gain an insight into the complex interactions that exist between bacteria, their environment, host immunity, other microbes and antimicrobial therapy, and in turn, may present opportunities for antimicrobial intervention (Rogers *et al.*, 2010a).

Evidence-based systems of determining antibiotic efficacy during the treatment

One way in which our increased ability to understand bacterial infections might facilitate a reduction in antibiotic use is through the development of better systems for determining the success or failure of a particular treatment. By providing early indications of treatment success, evidence-based systems of determining antibiotic efficacy during the treatment of an infection could lead to reduced antibiotic exposure, in turn, reducing the selective burden and slowing the emergence and spread of resistance. For example, culture-independent PCR-based assays can be used to enumerate viable bacterial cells in samples taken from the site of infection (Rogers *et al.*, 2010b). With calibration, this will provide a rapid and objective indication of treatment success.

The need for the introduction of such strategies is even more pronounced when dealing with chronic infections, where eradication of the pathogen may be challenging or even not realistically achievable. For example, antibiotics used in treating exacerbations of clinical symptoms in conditions such as cystic fibrosis lung infections still follow a standard 10–14 day high-dose course, derived from the principles of eradication in simple infections (Cystic Fibrosis Trust, 2009). Here, identifying appropriate microbiological outcomes, and determining empirically when these have been achieved, would provide a rationale for limiting antibiotic use to only that which is clinically beneficial (Rogers *et al.*, 2011). Given our co-existence with the array of microbes at many body sites, antimicrobial therapy can be better thought of as the prevention or favourable modification of the course of infectious disease (Patrick and Hutchinson, 2009).

Targeting bacterial behaviour

Being able to determine key characteristics of bacterial infections and track the way that these factors change during the course of infection and treatment may be only a start. As the mechanisms of infection increasingly become known, opportunities present themselves to influence, disrupt and even direct bacterial behaviour at the site of infections in ways that increase the efficacy of both clearance by the host immune response and by antibiotic therapy.

Such intervention is necessary given that the inefficiency of current treatments of bacterial infections is a key reason for levels of antibiotic usage. Despite bacterial strains being shown in many instances to be susceptible under *in vitro* conditions to the antibiotics being administered, high doses, for extended periods, and sometimes with several different antibiotics, are often necessary. Rather than resulting from the acquisition of active resistance traits by target bacterial populations, poor treatment efficacy *in vivo* is most commonly due to the adoption of growth strategies that support antibiotic tolerance. The two best recognized strategies that bacteria employ are the formation of biofilms and the creation of non-multiplying, persister subpopulations. The difficulties in developing novel antibiotics that are effective against such drug-tolerant subpopulations are clear. It may, however, be possible to induce bacteria present at the site of an infection to revert to less tolerant modes of growth, conferring a greater level of treatment efficacy with existing antibiotics. Crucially, such survival strategies are under the control of gene expression and, as such, are reversible. Further, their organization by bacterial populations requires communication and coordination, processes that can be interfered with and manipulated.

Here, we examine the ways in which these survival traits, which play a major role in the ability of bacteria in infections to withstand antibiotic treatment, can be disrupted to achieve greater antibiotic efficacy. The deployment of such behaviour manipulation promises to reinvigorate existing antibiotic therapies, in turn, reducing the rate at which resistance is emerging.

Persister cells

Bacteria present particularly in chronic infections are known to contain subpopulations of 'persister' cells (Lewis, 2007). Persisters make up a small fraction of the population but can be the only cells at the site of infection to survive treatment with high doses of bactericidal antibiotics. Their tolerance of antibiotics is mechanistically distinct from resistance, and is thought to stem primarily from the fact that these cells are largely inactive (Coates *et al.*, 2002; Hu *et al.*, 2005), and therefore protected against the reactive oxygen species that result from the action of most bactericidal antibiotics (Dworkin and Shah, 2010; Kohanski *et al.*, 2010).

From a clinical perspective, it has been suggested that 60% of human bacterial infections contain non-multiplying bacteria (Coates and Hu, 2008). The presence of such persister cells can therefore reseed the site of an infection following cessation of treatment. In addition, persisters can provide a reservoir of viable bacteria that can acquire resistance by random mutation or horizontal gene transfer, thereby promoting the generation of antibiotic-resistant mutants (Fauvart *et al.*, 2011).

It has been suggested that a bacterial population, including persisters, can be most effectively eradicated through periodic dosing of antibiotics (De Leenheer and Cogan, 2009), and, as such, a re-evaluation of drug delivery practices may be of benefit. However, the optimal protocol entails specific treatment and withdrawal durations, dependent on bacterial kinetics. Further, as pointed out by (Fauvart *et al.*, 2011), the practical utility of these predictions is limited by the need to experimentally determine model parameters, an extremely challenging task in *in vivo* situations.

Currently, the best hope of challenging persister cells is either by attempting to kill these cells 'directly' or by inducing them to revert from their persister phenotype so that currently available drugs are effective against them. The direct killing of persister cells, through for example, the use of an antibiotic active against persister cells would be an attractive option (Coates *et al.*, 2002; Coates and Hu, 2007), and could perhaps be deployed in combination with antibiotics that kill multiplying bacterial cells. However, the development of such compounds is hindered by a number of factors. The already limited number of molecular targets for antibiotics is further reduced in metabolically-inactive populations as many of their genes are down-regulated (Hu and Coates, 1999a,b; Beenken *et al.*, 2004; Johansen *et al.*, 2006). Further, the diversity of growth strategies and metabolic states mean that antibiotics that are effective against some subpopulations are still likely to leave others intact and so are able to re-establish infection. However, this is a feasible route. A recent screening effort resulted in the identification of compounds that almost exclusively kill non-replicating cells of the pathogen *Mycobacterium tuberculosis* (Bryk *et al.*, 2008). Despite this advance, the process of developing other such treatments is likely to be even more challenging than identifying new antibiotics that are effective against rapidly growing cells, something that is already fraught with difficulty.

While not yet fully understood, the mechanisms that govern the formation of persister populations are beginning to emerge (Mulcahy *et al.*, 2010; De Groote *et al.*, 2011). As we understand more the mechanisms involved, new potential drug targets to disrupt this behaviour are likely to be revealed. One approach is to develop strategies to resuscitate dormant persisters, rendering them sensitive to the lethal action of conventional antibiotics, or to prevent the formation of persister cells to begin with (Fauvart *et al.*, 2011). In order to do this, we need to understand the mechanisms that control this behaviour.

Targets for the disruption of persistence

Despite significant advances in our knowledge of persister regulation, the systems involved remain, in the most part, to be described. However, one mechanism that has been proposed for persister formation is increased expression of chromosomal toxin–antitoxin (TA) protein genes, where one protein acts as a toxin and the other cancels out its effect (Shah *et al.*, 2006; Lewis, 2007). These genes are typically located as pairs, usually in the same operon, forming TA

modules. Two such TA systems, encoded by the hipBA and tisAB loci in *E. coli*, have been studied in some depth.

The hipBA TA module consists of two genes, *hipA* and *hipB* (Moyed and Broderick, 1986; Black *et al.*, 1991). *hipA* encodes the toxin HipA, which phosphorylates the translation factor EF-Tu (Schumacher *et al.*, 2009), a process which is required for the induction of the persister state (Correia *et al.*, 2006). The hipB gene encodes an antitoxin which forms a tight complex with HipA, and represses the hipBA operon (Black *et al.*, 1991, 1994; Schumacher *et al.*, 2009). When unbound HipA exceeds a threshold level, growth arrest and entry into the persistent state is triggered (Rotem *et al.*, 2010) a process which can be reversed by overexpression of the antitoxin (Falla and Chopra, 1998; Keren *et al.*, 2004; Korch and Hill, 2006; Vazquez-Laslop *et al.*, 2006).

The SOS response is a stress response mechanism that has previously been implicated in persistence (Debbia *et al.*, 2001; Keren *et al.*, 2004; Dorr *et al.*, 2009) and is induced by DNA damage, such as that caused by fluoroquinolone antibiotics. As such, interference with this stress response may also be a potential drug target (Dorr *et al.*, 2010).

In each case, these mechanisms provide opportunities to develop drugs that promote transition from persister to actively growing states in bacteria causing infection. However, the degree to which these mechanisms might be relevant to multiple species is unclear (Dworkin and Shah, 2010). For example, while HipA and TisB represent the best studied persistence proteins, neither *hipA* nor *tisB* is present in the genomes of *M. tuberculosis*, *Pseudomonas aeruginosa* or *S. aureus* (Fauvart *et al.*, 2011). Further, screening of mutant libraries suggests that there are multiple, redundant mechanisms of persister formation (Hansen *et al.*, 2008; Dorr *et al.*, 2010).

An alternative approach is to target global regulators. Global regulators are better conserved among bacterial species, and a number of them have been linked to persistence (Korch *et al.*, 2003; Viducic *et al.*, 2006; Hansen *et al.*, 2008; Fauvart *et al.*, 2011) and have been suggested as potential drug targets (Li and Zhang, 2007; Lamarche *et al.*, 2008). However, because of their simultaneous involvement in additional processes, their suitability for this role remains to be determined.

Biofilms

One of the best recognized strategies by which bacteria are able to tolerate antibiotic exposure in the body is through the formation of biofilms. Biofilms are multicellular aggregate structures in which bacteria exhibit marked differences in growth rate, morphology and gene expression in comparison to planktonic cells of the same strain (Costerton *et al.*, 1995).

Depending on the nature of the infection, populations growing as biofilms are thought to be involved in more than 65% of human microbial infections (Potera, 1999). Biofilm growth is also linked to persister cells. Persister cells are present in bacterial biofilms, and it has been suggested that persisters are responsible in a significant part of the tolerance of cells in a biofilm to antibiotics (Spoering and Lewis, 2001; Roberts and Stewart, 2005). In addition to a reduced rate of growth and metabolic processes, biofilm cells are typically encased in an exopolysaccharide matrix that provides further protection against antibiotics. Biofilms are generally highly tolerant of standard agents and can be more than 1,000-fold more resistant than are planktonic cells (Brooun *et al.*, 2000). Dealing with biofilm-based growth therefore is important. This, in turn, supports drives to obtain new agents to deal with either the slow growing cells within the biofilm or to make cells in these structures more accessible to existing antibiotics.

The transition from the relatively antibiotic-susceptible planktonic mode of growth and the more resistant biofilm state is reversible. Bacteria within biofilms can undergo coordinated dispersal events in which attached biofilm cells convert to free-swimming planktonic bacteria (Webb *et al.*, 2003; Purevdorj-Gage *et al.*, 2005; Barraud *et al.*, 2006). It has been suggested that this process benefits bacteria by allowing single organisms to return to the liquid phase and colonize new habitats (Sauer *et al.*, 2004). A range of factors can drive change in regulation, including the sudden elevation or lowering of nutrient levels (Hunt *et al.*, 2004; Sauer *et al.*, 2004; Schleheck *et al.*, 2009), the induction of lysogenic bacteriophage (Lu and Collins, 2007) or in the context of the respiratory tract, infection by human respiratory viruses can modulate bacterial behaviour through their impact of epithelial cell behaviour (Chattoraj *et al.*, 2011).

Bacteria employ a range of other systems that modify the structures of their own biofilms (Karatan and Watnick, 2009) and several bacterial regulatory systems and active dispersal mechanisms have been linked to this transition (Boyd and Chakrabarty, 1995; Davey *et al.*, 2003; Hunt *et al.*, 2004; Sauer *et al.*, 2004; Gjermansen *et al.*, 2005; Rice *et al.*, 2009). The systems that bacteria use to regulate biofilm formation therefore present many potentially useful opportunities to direct biofilm dispersal. For example, the oral pathogen *Aggregatibacter actinomycetemcomitans* produces a protein that specifically hydrolyzes the glycosidic linkages of poly-β-1, 6-N-acetylglucosamine (PNAG) (Itoh *et al.*, 2005). This protein, dispersin B, can prevent biofilm formation and trigger biofilm detachment in any PNAG-producing bacterial species biofilms. Exposure to dispersin B in the presence of antibiotics (Donelli *et al.*, 2007; Izano *et al.*, 2007, 2008) or disinfectants such as triclosan results in synergistic biofilm removal and bacterial killing (Darouiche *et al.*, 2009). Dispersin B in combination with triclosan is now marketed in gel preparations for the treatment of wound and skin infections and for disinfection of medical devices, suggesting that combinations of antibiotics and enzymes that degrade the exopolysaccharide component of biofilms can represent a powerful tool for biofilm eradication in these settings (Landini *et al.*, 2010).

In *P. aeruginosa*, it has been shown that treatment of biofilm-growing cells with toxic compounds, such as heavy metals, results in detachment of biofilm cells, probably a defence response aimed at mobilizing bacterial cells. This process requires the environmental sensor BdlA, which can trigger degradation of the intracellular second messenger cyclic di-GMP (c-di-GMP), in turn resulting in the breakdown of the biofilm (Simm *et al.*, 2004; Morgan *et al.*, 2006; Thormann *et al.*, 2006; Schleheck *et al.*, 2009).

Such degradation of c-di-GM can also be triggered by cell damage caused by oxidate and nitrosative stress (Webb *et al.*, 2003), for example, as a result of high levels of NO (De Groote

and Fang, 1995; Barraud *et al*., 2006; 2009a). However, in addition to their damaging properties, reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) are involved in many signalling and regulatory pathways (Wagner *et al*., 2003). Barraud *et al*. (2009b) demonstrated that sublethal concentrations of NO reduce bacterial attachment, induce biofilm dispersal and enhance motility. Further, they showed that such exposure resulted in an increased susceptibility to antibiotics, both in planktonic and biofilm populations (Barraud *et al*., 2009a). The c-di-GMP internal signalling pathway disrupted by NO may be conserved across microbial species (Barraud *et al*., 2009a) and indeed, NO has been shown to induce dispersal in biofilms of several Gram-positive and Gram-negative species, as well as in mixed biofilms (Barraud *et al*., 2009b). Further, the development of NO-releasing silica nanoparticles (Hetrick *et al*., 2008; 2009) may allow more efficient delivery of NO to the site of infection.

Kolodkin-Gal *et al*. (2010) showed recently that prior to biofilm disassembly *Bacillus subtilis* produced a factor that prevented biofilm formation and could break down existing biofilms (Kolodkin-Gal *et al*., 2010). The factor was shown to be a mixture of D-leucine, D-methionine, D-tyrosine and D-tryptophan that could act at nanomolar concentrations. D-amino acid treatment caused the release of amyloid fibres that linked cells in the biofilm together. They also went on to demonstrate that D-amino acids also prevented biofilm formation by *S. aureus* and *P. aeruginosa*. As such, D-amino acids might prove widely useful in medical and industrial applications for the prevention or eradication of biofilms.

A number of other potential strategies to increase the proportion of bacteria present as planktonic cells within an infection have also been identified. Pan *et al*. (2011) reported the development of novel thiazolidiones derivatives that are both potent *Staphylococcus epidermidis* biofilm dispersants and efficient antibacterial agents, an ongoing process that may yield clinically applicable agents (Pan *et al*., 2011). The search for novel biofilm inhibitors has also targeted nucleotide biosynthesis. Uracil has been shown to influence all three known quorum sensing (QS) pathways (as explained further later) of *P. aeruginosa* (Attila *et al*., 2009; Ueda *et al*., 2009). Further, the anticancer uracil analogue, 5-fluorouracil, has been shown to repress biofilm formation, abolished QS phenotypes and reduced virulence (Attila *et al*., 2009; Ueda *et al*., 2009).

Inhibition of QS

Much of the coordinated behaviour exhibited by bacteria is governed by QS systems. These are based on the production and response to small diffusible molecules that act as signals. These molecules have been termed autoinducers (AIs). AIs are produced at basal levels, and their concentration increases with growth. Because the signals can diffuse through membranes, their concentration inside cells approximates the concentration in the environment. Upon reaching a critical concentration, the signal molecules can bind to and activate receptors inside bacterial cells. These receptors can then alter gene expression to activate behaviours that are beneficial under the particular condition encountered. This process is termed QS as it occurs in a cell density-dependent manner (Fuqua *et al*., 1994; 2001; Bassler, 1999; Antunes and Ferreira, 2009; Antunes *et al*., 2010).

A significant proportion of virulence traits, across a wide diversity of bacterial species, are controlled by QS (Antunes *et al*., 2010). Indeed, many of the mechanisms involved in both biofilm regulation and the formation of persister populations are controlled by QS systems (Hentzer *et al*., 2002; Manefield and Turner, 2002; Bjarnsholt *et al*., 2005; Persson *et al*., 2005; Rasmussen *et al*., 2005; Bjarnsholt and Givskov, 2008; Kayama *et al*., 2009; Moker *et al*., 2010).

Different bacterial species may produce different types of QS signals (Eberl, 1999), but they appear to adopt only two general mechanisms for detecting and responding to these signals (Dong *et al*., 2007). One general mechanism is represented by *N*-acyl-homoserine lactone (AHL)-dependent QS systems, in which the QS signal is detected by a cytosolic transcription factor. In the other mechanism, the QS signal, such as the autoinducing peptide (AIP) produced by *S. aureus*, is detected by a membrane-associated two-component response regulatory system (Dong *et al*., 2007). Most bacterial species appear to use one or other of these systems to modulate target gene expression, but in some cases, pathogens may employ both QS mechanisms for the same purpose (Waters and Bassler, 2005).

Therefore, interference with QS systems disrupts these survival strategies, rendering bacteria more sensitive to antibiotics. The process of interfering with QS systems is termed 'quorum quenching' (QQ) (Dong *et al*., 2001). There are a number of ways in which the disruption of QS systems may be achieved (reviewed in depth by (Hentzer and Givskov, 2003; Zhang and Dong, 2004; Dong *et al*., 2007; Sperandio, 2007; Uroz *et al*., 2009; Landini *et al*., 2010). These can be grouped into those strategies that inhibit QS signal generation, those that inhibit signal dissemination and those that inhibit signal reception, and have been reviewed in depth elsewhere (Hentzer and Givskov, 2003). Here, some brief examples of strategies used are given.

Knowledge about signal generation can be exploited to develop QS inhibitor (QSI) molecules that target signal generation. For example, S-adenosyl methionine (SAM), is an amino donor for generation of the homoserine lactone ring in the sysnthesis of AHLs signal molecules in the well-studied LuxR-LuxI QS system common among Gram-negative bacteria (Eberl, 1999; Hentzer and Givskov, 2003). Analogues of S-adenosylhomocysteine, S-adenosylcysteine and sinefungin, have been demonstrated to be potent inhibitors of AHL synthesis catalysed by the *P. aeruginosa* RhlI protein (Parsek *et al*., 1999).

QS can be inhibited by a decrease in the active signal-molecule concentration in the environment. This may occur as a consequence of a non-enzymatic reaction, for example, AHL signals are subject to alkaline hydrolysis at high pH values (Yates *et al*., 2002). In addition, many bacteria are able to degrade or metabolize AHL molecules (Dong *et al*., 2000; Leadbetter and Greenberg, 2000; Wang and Kuramitsu, 2005; Medina-Martinez *et al*., 2007; Han *et al*., 2010). As such, it may be possible to exploit naturally occurring systems, or their derivatives, for QS signal disruption at the site of infection.

Finally, QS signal transduction can be blocked by an antagonist molecule that either competes or interferes with

the native AHL signal for binding to receptor. Several reports describe the *in vitro* application of AHL analogues to achieve inhibition of the QS circuits of various bacteria (Schaefer *et al.*, 1996; Swift *et al.*, 1997; 1999; Zhu *et al.*, 1998). An example is the furanone compounds isolated from marine macro algae (Kjelleberg *et al.*, 1997). These natural compounds have been used to derive synthetic furanones that have enhanced QSI properties against *P. aeruginosa* (Manny *et al.*, 1997), and these have been shown to attenuate *P. aeruginosa* lung infections in mouse models (Wu *et al.*, 2004). Natural furanones have, however, been shown to enhance biofilm formation in *S. aureus* at subinhibitory concentrations, as well as being toxic for murine fibroblasts (Kuehl *et al.*, 2009), suggesting there is some way to go before such compounds could be employed therapeutically. Synthetic furanones have been shown to inhibit *Salmonella* biofilm formation at sub-inhibitory concentrations, although there is no evidence that furanones act on the currently known *Salmonella* QS systems (Janssens *et al.*, 2008).

The process of identifying new compounds capable of inhibiting QS has been further aided by the introduction of ultra-high-throughput screening strategies. For example, Muh *et al.* (2006) used a cell-based assay to screen a library of ~200 000 compounds for inhibitors of the LasR QS system in *P. aeruginosa*, identifying a number of inhibitory compounds. These inhibitors included a tetrazole with a 12-carbon alkyl tail, designated PD12, that had a IC_{50} of 30 nM, and a phenyl ring with a 12-carbon alkyl tail, designated V-06–018, that had an IC_{50} of 10 μM (Muh *et al.*, 2006). Both compounds resembled the acyl-homoserine lactone molecule that normally binds to LasR and were shown to be general inhibitors of QS (Muh *et al.*, 2006).

In addition to inhibiting the ability of bacteria to adopt strategies that render them less susceptible to antibiotic therapy, such as persistence and the formation of biofilms, the disruption of QS systems has the added advantage of interfering with the expression of a wide range of other virulence factors, as well as having the potential to prevent the induction of proinflammatory responses in host cells (Williams *et al.*, 2004; Shiner *et al.*, 2006; Jahoor *et al.*, 2008). Such, QSI provides the additional benefits of rendering bacteria more susceptible to clearance by the immune system, and making their presence at the site of the infection less damaging to the host.

Macrolide antibiotics

Certain macrolide antibiotics have been shown to act as QSIs at subinhibitory concentrations (Pechere, 2001; Tateda *et al.*, 2001; Nalca *et al.*, 2006). For example, erythromycin has been reported to suppress production of *P. aeruginosa* hemagglutinins, protease, hemolysin and AHL signals (Sofer *et al.*, 1999), and studies have shown that azithromycin affects QS-regulated virulence genes *in vitro* (Tateda *et al.*, 2001; Nalca *et al.*, 2006; Hoffmann *et al.*, 2007) and in *in vivo* models of disease (Hoffmann *et al.*, 2007). The molecular mechanism of QS inhibition by macrolides has not yet been identified, but it seems likely that they might only affect QS in an indirect fashion through interaction with their primary target, the ribosome (Landini *et al.*, 2010).

Skindersoe *et al.* (2008) recently screened 12 antibiotics for their QS-inhibitory activities at subinhibitory concentrations and found azithromycin, ceftazidime and ciprofloxacin each to be highly active (Skindersoe *et al.*, 2008). These findings suggest that in clinical contexts, such as the treatment of *P. aeruginosa* infections in the cystic fibrosis lung, where they are given at levels subinhibitory for *P. aeruginosa* (Howe and Spencer, 1997) the benefits associated with macrolide therapy (Howe and Spencer, 1997; Equi *et al.*, 2002; Saiman *et al.*, 2003) may result not from bacterial inhibition, but from interference with their communication systems. Further, this leads to the use of combination antibiotic therapy, where drugs are given both a sub-minimum inhibitory concentration (MIC) concentrations to inhibit strategies that reduce treatment efficacies, and above MICs to achieve maximal killing.

A role for bacteriophage?

Bacteriophages and their fragments kill bacteria (Borysowski *et al.*, 2006) and bacteriophage therapy was used in the former Soviet Union to treat infections (Sulakvelidze *et al.*, 2001), as well as in a range of contexts, including the poultry and cattle industries (Huff *et al.*, 2003; Doyle and Erickson, 2006; Fischetti *et al.*, 2006; Sheng *et al.*, 2006), aquaculture (Nakai and Park, 2002) and in sewage treatment (Withey *et al.*, 2005). Phage have also been modified to extend their natural host range (Scholl *et al.*, 2005) to express lethal genes to cause cell death (Heitman *et al.*, 1989; Hagens and Blasi, 2003; Westwater *et al.*, 2003; Hagens *et al.*, 2004; Brussow, 2005). As such, the development of effective phage therapies may help to lighten the load borne by antibiotics. Such strategies have a number of disadvantages, including difficulties in standardization and quality control, immunogenic responses and the induction of neutralizing antibodies when used systemically (Dabrowska *et al.*, 2004), and the potential to trigger toxic shock in response to massive bacterial lysis (Matsuda *et al.*, 2005), although this can be mitigated by the use of lysis-deficient phages, which can still kill bacteria (Matsuda *et al.*, 2005). Further, phages that are directly lethal to their bacterial hosts can select for phage-resistant bacteria in a short time (Summers, 2001; Hagens and Blasi, 2003; Hagens *et al.*, 2004).

Use of indwelling catheters is often compromised as a result of biofilm formation. However, the pre-treatment of hydrogel-coated catheters with bacteriophage has been shown to substantially reduce the ability of *S. epidermidis* and *P. aeruginosa* to form biofilms on them (Curtin and Donlan, 2006; Fu *et al.*, 2010).

However, in addition to using lytic phage to kill bacteria present at the site of infection, it is also possible to use them to modify bacterial behaviour in a way that increases the efficacy of antibiotic therapy. For example, Lu *et al.* (2009) engineered non-lytic filamentous phage to overexpress proteins that repress the SOS response (Lu *et al.*, 2009), a DNA repair system that confers an ability to tolerate antibiotics (Kohanski *et al.*, 2007). Infection with the phage enhanced the killing of both antibiotic-resistant and antibiotic-sensitive bacteria in both *in vitro* and *in vivo* model systems. There is scope to further develop phage that can disrupt additional

gene networks and therefore augment the efficacy of a range of antimicrobial treatments. Such strategies offer great promise in the treatment of challenging infections.

How likely is it that these approaches will yield results?

Do any of the systems above have real potential however? While some way from being clinically deployed, previous estimates have suggested that drugs targeting, for example, QS systems will reach the market in 2–7 years (Bjarnsholt and Givskov, 2008) are heartening considering the current slow pace of discovery of novel antibiotics. The ability to disrupt the expression of traits that protect against antibiotics, for example, through the use of QSIs, represents a major advance. However, determining whether such strategies are indeed effective in increasing the impact of conventional antibiotics *in vivo* is the next logical step in developing more effective treatment processes. As demonstrated by the benefits seen with the use of modified bacteriophage, this is something that is now being undertaken, and for a range of strategies. For example, Brackman *et al*. (2011) looked at the impact of tobramycin against *P. aeruginosa* and *Burkholderia cepacia* complex and clindamycin or vancomycin against *S. aureus*, either alone or in combination with QS inhibition (Brackman *et al*., 2011). In a number of *in vitro* and *in vivo* biofilm model systems, it was demonstrated that combined use of an antibiotic and a QSI generally resulted in increased killing compared with killing by an antibiotic alone and/or increased host survival following infection. Strategies such as these to disrupt coordinated bacterial behaviour have real potential to augment the efficacy of currently available antibiotic drugs. In this way, it may be possible to buy the time that is needed to develop additional strategies in the face of spreading antibiotic resistance.

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Conflicts of interest

None.

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